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ORIGINAL ARTICLE

Interactions of organic acids with vancomycin-resistant *Enterococcus faecium* isolated from community wastewater in Texas

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Keywords
acetic acid, citric acid, formic acid, lactic acid, molar minimum inhibitory concentrations, propionic acid, susceptibility, vancomycin-resistant *Enterococcus faecium*.

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Abstract

Aims: Investigate the interactions of organic acids (OAs), acetic, butyric, citric, formic, lactic and propionic acid against 50 Gram-positive vancomycin-resistant *Enterococcus faecium* (VRE) strains to determine whether pH, undissociated or dissociated acid forms correlate with bacterial inhibition.
Methods and Results: Concentrations of undissociated and dissociated OAs at the molar minimum inhibitory concentrations (MIC_Ms) of the VRE were calculated using the Henderson–Hasselbalch equation. The pH at the MIC_Ms of all VRE strains against acetic, butyric, formic and propionic acids was similar, 4·66 ± 0·07, but there was a 1·1 pH unit difference for all six OAs. Inhibition of VRE by all six OAs did not appear to be solely dependent on pH or on the undissociated OA species. The inhibition of VRE by all six dissociated acids was within $\Delta = 3{\cdot}1$ mmol l^{-1}.
Conclusions: Vancomycin-resistant *Enterococcus faecium* inhibition correlated with the dissociated OA species. A small decrease in the concentration of the dissociated OAs from optimum may result in allowing VRE strains to escape disinfection.
Significance and Impact of the Study: When an OA is used to disinfect VRE strains, the concentration of the dissociated OA should be carefully controlled. A concentration of at least 20 mmol l^{-1} dissociated OA should be maintained when disinfecting VRE.

Introduction

Vancomycin-resistant enterococci (VRE), which causes human infections is found throughout the world, and is frequently found in hospitals and health care facilities (Fridkin *et al.* 1998; Kim *et al.* 1999; Bonten *et al.* 2001; Tomita *et al.* 2002; Tacconelli *et al.* 2004; Novais *et al.* 2005a; Isenman *et al.* 2016). The infections resulting from VRE are associated with higher treatment costs, prolonged morbidity and greater mortality (Calfee *et al.* 2003). The prevalence of VRE varies widely from 0 to 45% of the *Enterococcus faecium* isolated in European countries (EARS 2014); however, in 2000, the prevalence of enterococcal infections resistant to vancomycin in the United States as determined by the National Nosocomial Infectious Surveillance (NNIS) System was 26·3%, which at the time was a 31% increase from 1995 (NNIS 2001). In the United States, up to 3% of hospital infections are due to VRE (Sievert *et al.* 2013). These fermentative and aerotolerant Gram-positive enterococci bacteria are ubiquitous in nature and are found in the environment (Byappanahalli *et al.* 2012), in association with animals and vegetables (Klein 2003; Hammerum *et al.* 2010; Ben Said *et al.* 2016), and can be found in food products

(McGowan *et al.* 2006). Due to the growth characteristics of enterococci, this organism is often used as an indicator of faecal water contamination (Boehm and Sassoubre 2014; NARS 2018).

The VRE used in this study were not obtained from hospital or health care facility sources, but were isolated from human wastewater effluents at a nonclinical semiclosed agri-food system with restricted access, housing a long-term permanent worker population in Texas (Poole *et al.* 2005). Human wastewater effluents are known to be a source of exposure to VRE (Poole *et al.* 2005; Talebi *et al.* 2008; Varela *et al.* 2013) as well as the effluents of wastewater treatment plants (Varela *et al.* 2013; Goldstein *et al.* 2014), and also the reusable effluent from wastewater treatment plants (Goldstein *et al.* 2014).

Biocides are commonly used to control bacteria and are often used in the form of antiseptics and disinfectants (Beier *et al.* 2017a). Biocides are 'substances and preparations containing one or more active compounds intended to inactivate or exert a controlling effect on harmful microorganisms' (Ruiz and Alvarez-Ordóñez 2017). Just as bacteria can resist the action of antimicrobial agents through decreased uptake or increased efflux (McDermott *et al.* 2003), the efficacy of a biocide may be reduced by low permeability of the cell wall or active efflux mechanisms (Fraise 2002). One group of chemicals used to control bacteria is the organic acids (OAs), which are considered to be biocides (Ruiz and Alvarez-Ordóñez 2017). In a critical step during the processing of animals into food products, OAs are used to wash the animal carcasses to remove surface bacteria. The OAs used during this critical step are often acetic (PSU *et al.* 2005; Raftari *et al.* 2009, 2011), citric (PSU *et al.* 2005), formic (Raftari *et al.* 2009), lactic (Epling *et al.* 1993; Castillo *et al.* 2001; PSU *et al.* 2005; Reynolds 2005; Raftari *et al.* 2009, 2011) and propionic acids (Raftari *et al.* 2009, 2011).

Over the years many researchers have believed that the inhibition of bacteria by OAs was dependent on pH or the undissociated acid species (Sofos and Busta 1981; Blocher *et al.* 1982; Ray and Sandine 1992; Leeson 2007); however, the mechanism of inhibition of bacteria by pH and OAs is not understood (Presser *et al.* 1998). The results from previous studies with Gram-negative pathogenic bacteria against OAs clearly show that pH and the levels of undissociated acids do not correlate with the molar minimum inhibitory concentrations (MIC_Ms) of the OAs. Molar values have been used for the MICs when comparing pH, and the undissociated or dissociated acid forms of the OAs because it allows an equivalent comparison of MIC results for acids with different molecular weights (Beier *et al.* 2013). In studies of the interactions of OAs with *Escherichia coli* O157:H7 (Beier *et al.* 2013), *Pseudomonas aeruginosa* (Beier *et al.* 2014), non-O157 Shiga toxin-producing *E. coli* (non-O157 STECs) (Beier *et al.* 2016), *Salmonella* enterica serovars (Beier *et al.* 2017b) and *Campylobacter coli* (Beier *et al.* 2018), the levels of dissociated acids were closely correlated with the MIC_Ms of the bacteria in all studies. It also has previously been shown that a fully dissociable acid will cause the disintegration of the bacterial LPS layer (Alakomi *et al.* 2000). A small decrease in the concentration of the dissociated acids may result in a large number of bacteria escaping disinfection (Beier *et al.* 2013, 2014, 2016, 2017b, 2018).

In this study, we describe the interactions of six different OAs with 50 VRE strains, which were obtained in an earlier study to assess VRE shedding from a largely nonclinical community population in the United States (Poole *et al.* 2005). OA susceptibility studies of the 50 VRE strains against acetic, butyric, citric, formic, lactic and propionic acids were conducted here. The pH and the undissociated and dissociated OA species concentrations evaluated at the MIC_Ms of the VRE strains are compared.

Materials and methods

Bacterial strains

Fifty VRE strains were previously isolated at seven locations between Huntsville, TX, USA and South of Houston, TX, USA and obtained from human wastewater effluents in a nonclinical semiclosed agri-food system with restricted access, housing a long-term permanent worker population (Poole *et al.* 2005). Bacterial strains were then stored at −76°C until used and reconstituted as previously described (Beier *et al.* 2005).

Chemicals

Acetic acid was obtained from EM Science (Gibbstown, NY). Butyric, citric, formic and propionic acids were obtained from Sigma-Aldrich (Milwaukee, WI). Lactic acid was obtained from Alfa Aesar (Wad Hill, MA). Working solutions of OAs were diluted with reverse osmosis water and filter sterilized using a 0·2 μm × 25 mm syringe filter (No. 431224; Corning Inc., Corning, NY).

Organic acid susceptibility testing

The VRE MICs obtained against six OAs were determined by broth microdilution according to the Clinical and Laboratory Standards Institute (CLSI 2012). The lowest concentration of a chemical compound that showed no visible growth was determined as the MIC (Andrews

2001). The OA susceptibility studies were carried out similar to previous studies of other bacterial human pathogens (Beier *et al.* 2013, 2014, 2016, 2017b, 2018). Briefly, Mueller–Hinton broth (50 μl) (Becton Dickinson and Company, Sparks, MD) was added to each well in column 2 through the wells in column 12 of a 96-well U-bottom Greiner bio-one microplate (#82050-626; VWR, Houston, TX). The OA dilutions in the wells were made by adding 50 μl of each standard OA solution to the wells in column 1 and column 2, and the solutions in the wells of column 2 were diluted 1 : 2 across the 96-well microplate through the wells in column 11, and the wells in column 12 were used as the positive control (Beier *et al.* 2017b). Several well-isolated colonies were selected from a sheep blood agar plate (BVA Scientific Inc., San Antonio, TX), and transferred to a 5-ml Sensititre® demineralized water tube (Remel, Lenexa, KS) and adjusted to a 0·5 McFarland Standard using a Nephelometer® (TREK Diagnostic Systems Ltd, East Grinsted, UK). This bacterial solution (100 μl) was transferred into a tube containing 11 ml Sensititre Mueller–Hinton broth with TES buffer (Remel) to give an inoculum of approximately $1{\cdot}8 \times 10^6$ CFU per ml. Then, 50 μl of the VRE inoculum was added to the solution in each well of the 96-well plates resulting in a total volume of 100 μl per well. The plates were covered with a plastic adhesive sealing film, SealPlate® (EXCEL Scientific Inc., Victorville, CA) and incubated for 20 h at 37°C. Growth in the wells was visually observed using a SensiTouch® imaging system (TREK Diagnostic Systems Ltd). *Enterococcus faecalis* ATCC 29212 was used as a control organism for OA susceptibility testing.

The following concentrations of OAs were tested: acetic acid, 32–32 768 μg ml^{-1}; butyric acid, 16–16 384 μg ml^{-1}; citric acid, 16–16 384 μg ml^{-1}; formic acid, 16–16 384 μg ml^{-1}; lactic acid, 8–8192 μg ml^{-1} and propionic acid, 32–32 768 μg ml^{-1}.

Solution pH determination at the VRE MICs in the 96-well plates

The pH was obtained as previously described (Beier *et al.* 2017b). Briefly, the pH was determined in samples using an Orion 3 STAR benchtop pH meter with a ROSS Ultra, glass combination pH electrode (Thermo Fisher Scientific, Chelmsford, MA). The solutions from 16 wells (1600 μl) from 96-well microplates at the same MIC value, for all MICs, and for all six OAs were combined in sterile 5-ml microtubes (Argos Technologies, Inc., Vernon Hills, IL). Each pH determination at each MIC was conducted in triplicate samples, and then the means and standard deviations were determined.

Calculation of the ratio of undissociated to dissociated acids

The Henderson–Hasselbalch equation was used to calculate the molar concentration of the conjugate base and undissociated weak acid (Helmenstine 2018):

$$\mathrm{pH} = \mathrm{pK_a} + \log([\mathrm{A^-}]/[\mathrm{HA}]) \quad (1)$$

where the $\mathrm{pK_a} = -\log_{10}$ of the acid dissociation constant (K_a), $[A^-]$ = the molar concentration of the conjugate base (or dissociated weak acid) and [HA] = the molar concentration of the undissociated weak acid (Helmenstine 2018). Upon rearrangement of the Henderson–Hasselbalch equation, the ratio of undissociated to dissociated acid can be obtained (Blocher *et al.* 1982):

$$\mathrm{ratio} = [\mathrm{HA}]/[\mathrm{A^-}] = 1/10^{\mathrm{pH}-\mathrm{pK_a}} \quad (2)$$

When the pK_a of an OA and the pH of the solution at each MIC are known, then the ratio of the molar undissociated to dissociated acid can be calculated at these MICs. The pK_a for acetic, butyric, citric, formic, lactic and propionic acid is 4·75, 4·82, 3·14, 3·75, 3·86 and 4·87 respectively. The ratio can then be used to calculate the concentrations of the undissociated and dissociated acid species when the molar concentration of the OA is known at the MICs (Beier *et al.* 2013, 2014, 2016, 2017b, 2018).

Results

Measured VRE MICs against the organic acids

The MICs and MIC_Ms obtained for 50 high-level VRE strains compared with a vancomycin-susceptible *E. faecium* CF3 1.3 (Corrier *et al.* 1995) against the OAs tested here are shown in Table 1. The VRE MICs obtained for acetic and formic acid resulted in a single MIC for each acid, while MICs for the other four acids, butyric, citric, lactic and propionic, resulted in a range of MICs for each acid.

Table 2 presents the median, mode, range and 90th percentile for both the VRE MICs and the MIC_Ms for each OA. It is interesting that the median MIC_M values for butyric, citric, formic and lactic acid are very similar, while the MIC_M values for propionic and acetic acid are much higher. The ranges for butyric, lactic and propionic acid have the highest values while the ranges for citric and formic acid are lower, and are much lower than the other acids.

Measured pH at the VRE MICs against the organic acids

The pH was determined at all VRE MIC_Ms for all strains ($n = 50$) against each of the six OAs, and the VRE MIC_Ms for an individual OA were combined into a single

Table 1 Organic acid MICs and MIC_Ms for VRE strains isolated from community wastewater in Texas*

Organic acids	MIC ($\mu g\ ml^{-1}$)	MIC_M ($mmol\ l^{-1}$)	No. of VRE	CF3 1.3
Acetic acid	2048	34·10	50	1
Butyric acid	4096	46·49	40	1
	2048	23·24	10	–
Citric acid	4096	21·32	32	1
	2048	10·66	17	–
	1024	5·33	1	–
Formic acid	1024	22·25	50	1
Lactic acid	4096	45·47	2	–
	2048	22·74	47	1
	1024	11·37	1	–
Propionic acid	4096	55·29	1	–
	2048	27·65	40	1
	1024	13·82	9	–

MIC_Ms, molar minimum inhibitory concentrations; VRE, vancomycin-resistant *Enterococcus faecium*.
*Susceptibility profiles of 50 high-level vancomycin-resistant *E. faecium* (Poole *et al.* 2005) compared with *E. faecium* CF3 1.3 susceptible to vancomycin (Corrier *et al.* 1995).

Table 2 Central tendency of the organic acid MICs and MIC_Ms for 50 VRE strains isolated from community wastewater in Texas

Organic acid	Median	Mode	Range	90th Percentile
Acetic acid				
MIC ($\mu g\ ml^{-1}$)	2048	2048	2048	2048
MIC_M ($mmol\ l^{-1}$)	34·1	34·1	34·1	34·1
Butyric acid				
MIC ($\mu g\ ml^{-1}$)	2048	4096	2048–4096	4096
MIC_M ($mmol\ l^{-1}$)	23·24	46·49	23·24–46·49	46·49
Citric acid				
MIC ($\mu g\ ml^{-1}$)	4096	4096	1024–4096	4096
MIC_M ($mmol\ l^{-1}$)	21·32	21·32	5·33–21·32	21·32
Formic acid				
MIC ($\mu g\ ml^{-1}$)	1024	1024	1024	1024
MIC_M ($mmol\ l^{-1}$)	22·25	22·25	22·25	22·25
Lactic acid				
MIC ($\mu g\ ml^{-1}$)	2048	2048	1024–4096	2048
MIC_M ($mmol\ l^{-1}$)	22·74	22·74	11·37–45·47	22·74
Propionic acid				
MIC ($\mu g\ ml^{-1}$)	2048	2048	1024–4096	2048
MIC_M ($mmol\ l^{-1}$)	27·65	27·65	13·82–55·29	27·65

MIC_Ms, molar minimum inhibitory concentrations; VRE, vancomycin-resistant *Enterococcus faecium*.

group, for a total of six different groups. The pH values obtained at the VRE MIC_Ms for the six OAs are graphically shown in Fig. 1. Each pH data point is the mean and standard deviation of triplicate samples and next to each data point on the graph is depicted the number of strains associated at each MIC_M. There is a 1·1 pH unit

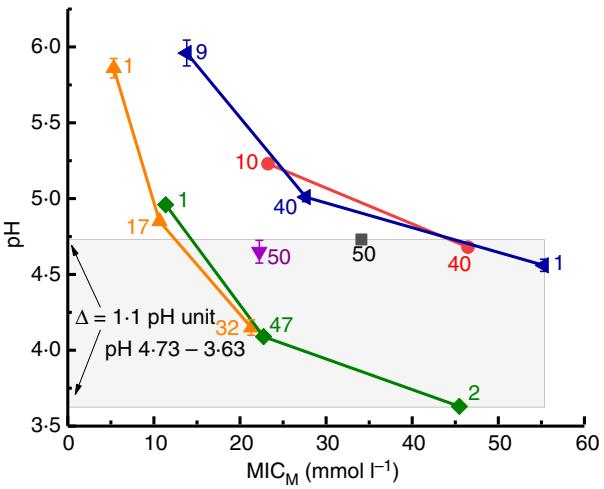

Figure 1 pH at the MIC_Ms for 50 vancomycin-resistant *Enterococcus faecium* strains against acetic (■), butyric (●), citric (▲), formic (▼), lactic (◆) and propionic (◀) acids. The number of strains is shown next to each data point. Each data point is the mean and standard deviation of triplicate samples. [Colour figure can be viewed at wile yonlinelibrary.com]

difference between 100% of the VRE strains inhibited by all six OAs. The pH at the MIC_M for 100% of the strains against acetic, butyric, formic and propionic acids was 4·73, 4·68, 4·65 and 4·56, respectively, having an average pH of 4·66 ± 0·07. The pH of these four OAs at the MIC_M for 100% of all 50 VRE are exceptionally close, and the pH of these four OAs may be directly involved with inhibition. But the pH value at the MIC_M for 100% of the VRE strains against citric and lactic acids was 4·15 and 3·63, respectively.

Undissociated organic acid concentrations calculated at the VRE MIC_Ms

The undissociated OA concentrations of acetic, butyric, citric, formic, lactic and propionic acids at the MIC_Ms of the VRE strains are shown in Fig. 2. These undissociated OA concentrations were obtained by using the Henderson–Hasselbalch equation and were calculated using the determined pH and the known OA concentration at the MIC_Ms. An undissociated citric acid level of 1·9 $mmol\ l^{-1}$ was required to inhibit all 50 VRE strains. A level of 37·11 $mmol\ l^{-1}$ undissociated propionic acid was required to inhibit the same 50 VRE strains. A level of 17·44, 26·96, 2·49 and 28·62 $mmol\ l^{-1}$ of undissociated acetic, butyric, formic and lactic acid, respectively, was required to inhibit the same VRE strains. The overall range of inhibition for 100% of the VRE strains against all six OAs ranged from 1·9 $mmol\ l^{-1}$ citric acid to 37·11 $mmol\ l^{-1}$ propionic acid for an undissociated acid difference of 35·21 $mmol\ l^{-1}$ (Fig. 2).

Dissociated organic acid concentrations calculated at the VRE MIC_Ms

The calculated dissociated OA concentrations of acetic, butyric, citric, formic, lactic and propionic acids at the MIC_Ms of the 50 VRE strains are shown in Fig. 3. The molar-dissociated OA concentrations at the MIC_Ms for 100% of the VRE strains against all six OAs are encompassed by the shaded band extending from 16·66 to 19·76 mmol l^{-1} (Fig. 3). The shaded band represents a Δ = 3·1 mmol l^{-1} difference between the MIC_M of 100% of the VRE strains inhibited by dissociated acetic acid and 100% of the strains inhibited by dissociated formic acid with the highest dissociated acid concentration level of 19·76 mmol l^{-1}. The other four dissociated OAs, including butyric, citric, lactic and propionic acids, inhibit 100% of the VRE strains within this small Δ = 3·1 mmol l^{-1} window. The comparison of pH and dissociated acid concentrations for all six OAs is shown in Fig. S1.

Discussion

Vancomycin-resistant *Enterococcus faecium* are found as environmental contaminants (Kühn *et al.* 2005; Novais *et al.* 2005b; Nilsson *et al.* 2009) in waste water effluents (Poole *et al.* 2005; Talebi *et al.* 2008; Varela *et al.* 2013), in farm animals (Giraffa 2002; Kühn *et al.* 2005; Hammerum 2012; Nilsson 2012) and in food products (Gambarotto *et al.* 2001; Giraffa 2002; Nilsson 2012). Enterococci can survive heat processing, especially if they are present in high numbers (Franz *et al.* 1999) as noted by the spoilage of pasteurized canned hams (Houben 1982; Magnus *et al.* 1986). Due to this heat tolerance, enterococci can survive being cooked to a temperature of 68°C for 30 min (Gordon and Ahmad 1991; Giraffa 2002); therefore, one then must depend upon disinfectants (Beier *et al.* 2008) and biocides like the OAs to eliminate VRE in the food chain. Decontamination strategies are often based upon pH (Shaheen *et al.* 2007), and many researchers have believed that the inhibition of bacteria by OAs is dependent on pH or the undissociated acid species (Sofos and Busta 1981; Blocher *et al.* 1982; Ray and Sandine 1992; Leeson 2007). But we have previously shown that Gram-negative pathogenic bacteria are inhibited by dissociated OAs, and not by pH or the undissociated acids alone (Beier *et al.* 2013, 2014, 2016, 2017b, 2018). Here, we investigate the effects of pH, undissociated OAs and dissociated OAs against Gram-positive VRE to evaluate the characteristic of OAs that result in VRE inhibition.

The MIC_M for the greatest amount of VRE strains against acetic and propionic acids was equivalent to the MIC_M obtained for the greatest amount of non-O157 STEC strains (Beier *et al.* 2016) and *C. coli* strains (Beier *et al.* 2018) against acetic and propionic acids. The MIC_M for the greatest amount of VRE strains against citric and lactic acids was equivalent to the MIC_M obtained for the greatest amount of O157:H7 strains (Beier *et al.* 2013)

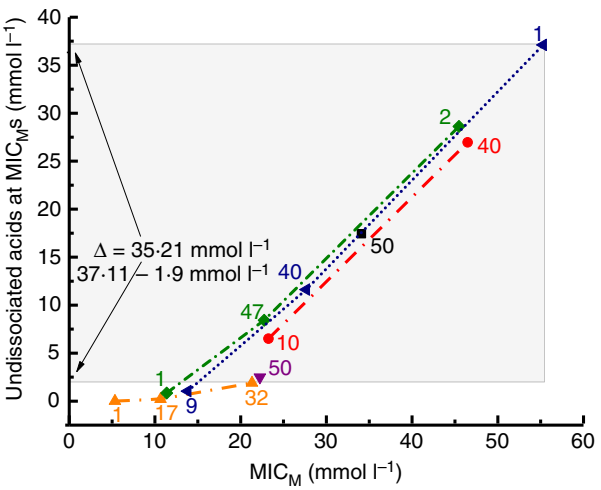

Figure 2 Concentration (mmol l^{-1}) of the undissociated acids at the molar minimum inhibitory concentrations (MIC_Ms) of 50 vancomycin-resistant *Enterococcus faecium* strains against acetic (■), butyric (●), citric (▲), formic (▼), lactic (◆) and propionic (◀) acids. The shaded band depicts the difference between the undissociated propionic acid and citric acid concentrations required for disinfection of 100% of all the strains, Δ = 35·21 mmol l^{-1}. The number of strains is shown next to each data point. [Colour figure can be viewed at wileyonlinelibrary.com]

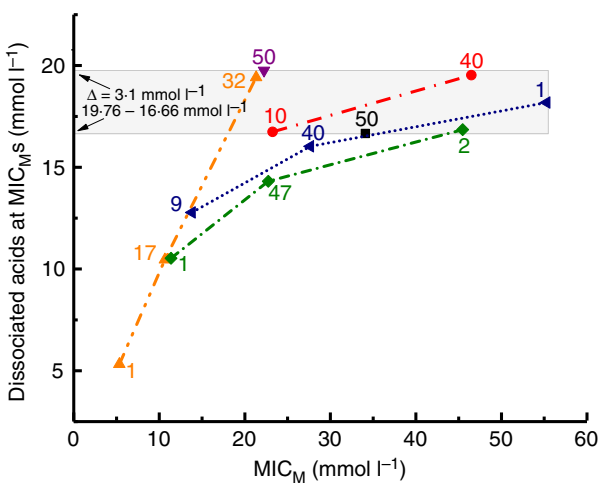

Figure 3 Concentration (mmol l^{-1}) of the dissociated acids at the molar minimum inhibitory concentrations (MIC_Ms) of 50 vancomycin-resistant *Enterococcus faecium* strains against acetic (■), butyric (●), citric (▲), formic (▼), lactic (◆) and propionic (◀) acids. The shaded band depicts the difference between the dissociated formic acid and acetic acid concentrations required for disinfection of 100% of all the strains, Δ = 3·1 mmol l^{-1}. The number of strains is shown next to each data point. [Colour figure can be viewed at wileyonlinelibrary.com]

and non-O157 STEC strains (Beier *et al.* 2016) against citric and lactic acids. There was a higher VRE MIC_M for citric and lactic acids than *C. coli* MIC_Ms for these same acids (Beier *et al.* 2018). There also was a greater amount of VRE strains with a higher butyric acid MIC_M than the MIC_M obtained for the greater amount of *C. coli* strains (Beier *et al.* 2018). It was interesting that the vancomycin-susceptible CF3 1.3 strain produced an MIC against each acid that was equivalent to the most abundant VRE MIC against each acid. Overall, lower molar amounts of citric, formic and lactic acids were required to inhibit the same 50 VRE compared with acetic, butyric and propionic acids.

The pH at the MIC_Ms of 100% of the VRE strains against all OAs showed a difference of 1·1 pH units. We have previously reported pH differences in inhibition of Gram-negative bacteria by OAs. A 0·56 pH unit difference was seen at the MIC_Ms when OAs inhibited 98% of 344 *E. coli* O157:H7 strains (Beier *et al.* 2013). About 98% of 175 *P. aeruginosa* strains showed a 0·98 pH unit difference for inhibition by OAs (Beier *et al.* 2014). There was a 0·99 pH unit difference at the MIC_Ms when OAs inhibited 100% of 138 non-O157 STEC strains (Beier *et al.* 2016). A 1·1 pH unit difference was seen when four OAs inhibited 100% of 145 *Salmonella* strains (Beier *et al.* 2017b); however, an average 1·76 pH unit difference was observed at the MIC_Ms for 6 OAs when inhibiting 111 *C. coli* strains (Beier *et al.* 2018). The data demonstrate that the Gram-positive VRE, as well as all the Gram-negative bacteria previously investigated, appears not to depend solely on pH for bacterial inhibition, as suggested by others (Blocher *et al.* 1982), but inhibition must depend on some other aspect of these acids. If pH was the primary cause of bacterial inhibition, then one would expect that the MIC_Ms for the same bacteria for each different OA would be at the same pH. Although in this study, four of six OAs inhibit the same 50 VRE at essentially the same pH, and in these cases pH appears to be directly involved with VRE inhibition by acetic, butyric, formic and propionic acids. However; there remains a 1·1 pH unit difference between all six OAs for the inhibition of 100% of the VRE tested, suggesting that citric acid and lactic acid inhibition of VRE may depend on some other aspect of the acids.

Following evaluation of the VRE for inhibition with respect to the undissociated acid species, it is clear that there is no association of the undissociated acids with VRE inhibition. All 50 VRE strains were inhibited by all 6 OAs over $\Delta = 35{\cdot}21$ mmol l^{-1} undissociated acids with the highest undissociated acid value being 37·11 mmol l^{-1}. The results obtained here with VRE are similar to those results previously observed with Gram-negative food pathogens. Inhibition of 100% of 175 *P. aeruginosa* strains required 2·53 mmol l^{-1} undissociated citric acid and 21·65 mmol l^{-1} undissociated acetic acid at the MIC_Ms, which resulted in a difference of 19·12 mmol l^{-1} undissociated OAs for inhibition (Beier *et al.* 2014). Inhibition of 98·3% of 344 *E. coli* O157:H7 strains required 2·86 mmol l^{-1} undissociated citric acid and 50·63 mmol l^{-1} undissociated acetic acid at the MIC_Ms for a difference of 47·77 mmol l^{-1} undissociated OAs (Beier *et al.* 2013). Inhibition of 100% of 138 non-O157 STECs strains required 2·2 mmol l^{-1} undissociated citric acid and 49·11 mmol l^{-1} undissociated acetic acid at the MIC_Ms for a difference of 46·91 mmol l^{-1} undissociated OAs (Beier *et al.* 2016). Inhibition of 100% of 145 *Salmonella* strains required 2·29 mmol l^{-1} undissociated citric acid and 19·0 mmol l^{-1} undissociated acetic acid at the MIC_Ms for a difference of 16·71 mmol l^{-1} undissociated OAs (Beier *et al.* 2017b). The inhibition of 100% of 111 *C. coli* strains required 0·024 mmol l^{-1} undissociated citric acid and 39·93 mmol l^{-1} undissociated acetic acid at the MIC_Ms, which is a difference of 39·91 mmol l^{-1} across all six OAs for the inhibition of the same strains (Beier *et al.* 2018). In these previous cases, as in this study, the undissociated OA concentration levels at bacterial inhibition did not correlate with the MIC_Ms.

The inhibition of 100% of VRE strains by all dissociated OAs ($\Delta = 3{\cdot}1$ mmol l^{-1}) was by far a much smaller concentration range than observed for the undissociated acids ($\Delta = 35{\cdot}21$ mmol l^{-1}). In previous studies, the inhibition of 98·3% of 344 *E. coli* O157:H7 strains by dissociated lactic acid (19·36 mmol l^{-1}) and propionic acid (13·825 mmol l^{-1}) resulted in a $\Delta = 5{\cdot}54$ mmol l^{-1} (Beier *et al.* 2013). Inhibition of 97·7% of 175 *P. aeruginosa* strains by dissociated lactic acid (21·91 mmol l^{-1}) and acetic acid (9·98 mmol l^{-1}) resulted in a $\Delta = 11{\cdot}93$ mmol l^{-1} (Beier *et al.* 2014). Inhibition of 100% of 138 non-O157 STEC strains by dissociated citric acid (19·12 mmol l^{-1}) and lactic acid (12·93 mmol l^{-1}) resulted in a $\Delta = 6{\cdot}19$ mmol l^{-1} (Beier *et al.* 2016). Inhibition of 100% of 145 *Salmonella* strains by dissociated citric acid (19·03 mmol l^{-1}) and propionic acid (13·67 mmol l^{-1}) resulted in a $\Delta = 5{\cdot}36$ mmol l^{-1} for inhibition of all strains (Beier *et al.* 2017b); and inhibition of 100% of 111 *C. coli* strains by dissociated butyric acid (22·56 mmol l^{-1}) and citric acid (10·64 mmol l^{-1}) resulted in a $\Delta = 11{\cdot}92$ mmol l^{-1} (Beier *et al.* 2018). Since both *P. aeruginosa* and *C. coli* utilize a number of different carboxylate anions, the dissociated OAs that have these carboxylate anions require higher levels to inhibit these bacteria (Beier *et al.* 2014, 2018).

The pH at the MIC_Ms of all VRE strains against acetic, butyric, formic and propionic acids was extremely close at the pH of $4{\cdot}66 \pm 0{\cdot}07$. The inhibition of VRE by these four OAs appears to be directly involved with the pH.

But for VRE inhibition by all six OAs there was a 1·1 pH unit difference. Therefore, inhibition of VRE strains by all six OAs was not solely dependent on pH or on the concentration of undissociated OAs. The concentration of dissociated acetic, butyric, citric, formic, lactic and propionic acids correlated with the MIC_Ms of 100% of the 50 VRE strains over a small $\Delta = 3{\cdot}1$ mmol l^{-1} range for all dissociated acids. One may expect that a large number of bacteria could escape disinfection as a result of only a small drop in the concentration of a dissociated OA. Therefore, an OA carcass wash or other disinfection uses of OAs may not provide the expected elimination of surface bacteria if the concentration levels of the dissociated OA used is not carefully controlled. A concentration of dissociated acetic, butyric, citric, formic, lactic and propionic acids of at least 20 mmol l^{-1} should be maintained when disinfecting VRE bacteria.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Data availability

All relevant data are within the paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. pH at the MIC_Ms of the 50 VRE strains against the dissociated forms of the six organic acids (OAs), acetic (■), butyric (●), citric (▲), formic (▼), lactic (◆) and propionic (◀) acids.